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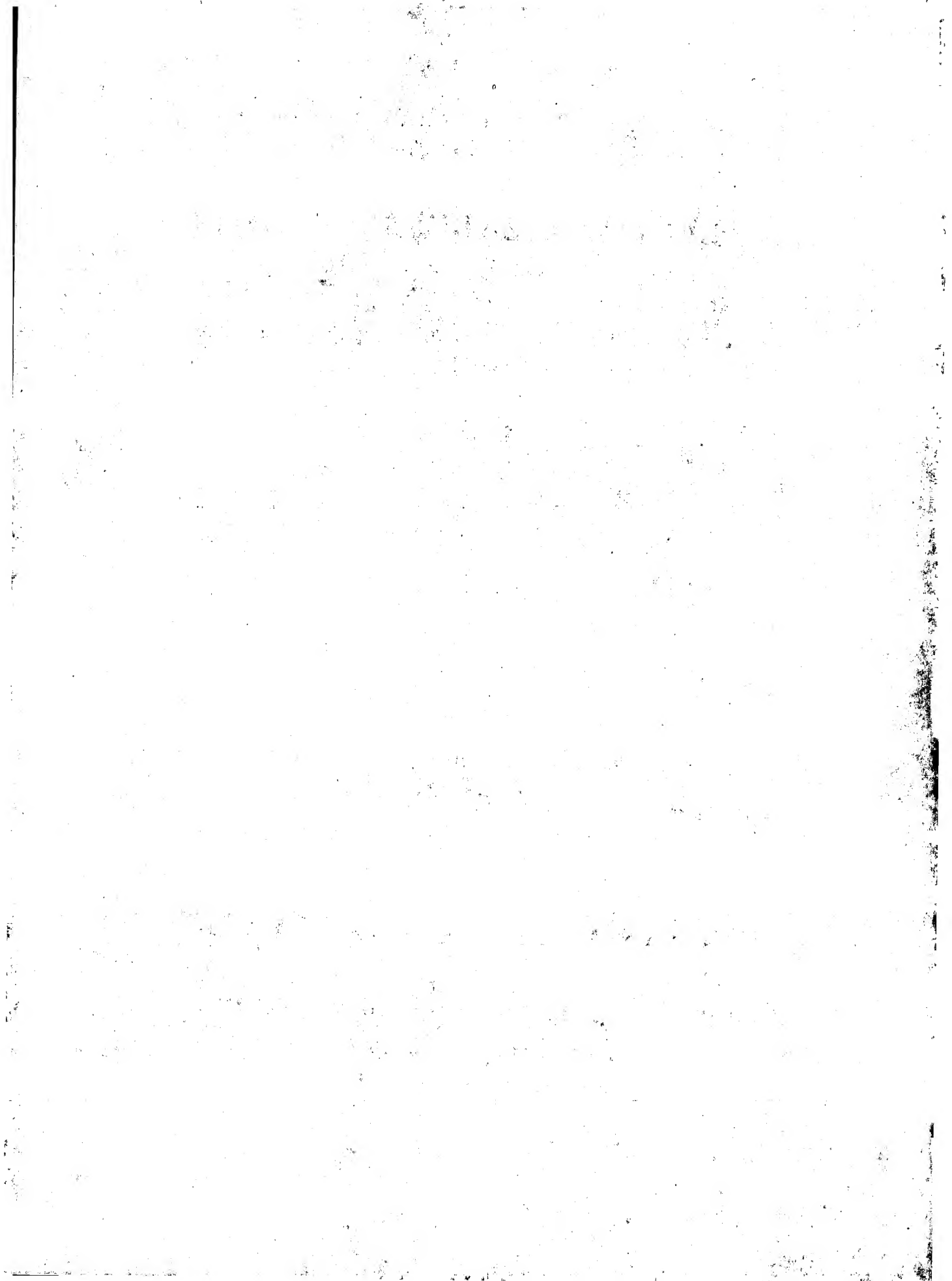
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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
29 November 2001 (29.11.2001)

PCT

(10) International Publication Number
WO 01/89542 A2

- (51) International Patent Classification⁷: **A61K 35/78**,
A61P 35/00, 29/00, 9/00 // (A61K 35/78, 35:74, 33:04,
31:375, 31:355, 31:015)
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- (21) International Application Number: PCT/EP01/05841
- (74) Agent: **LAUDIEN, Dieter**; Boehringer Ingelheim GmbH,
55216 Ingelheim am Rhein (DE).
- (22) International Filing Date: 22 May 2001 (22.05.2001)
- (81) Designated States (national): CA, CN, ID, JP, MX, US.
- (25) Filing Language: English
- (84) Designated States (regional): European patent (AT, BE,
CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC,
NL, PT, SE, TR).
- (26) Publication Language: English
- (30) Priority Data:
60/206,945 25 May 2000 (25.05.2000) US
- Published:
— without international search report and to be republished
upon receipt of that report
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(CH).
- For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.



WO 01/89542 A2

(54) Title: METHOD FOR IMPROVING THE CELL PROTECTION

(57) Abstract: The invention relates to a method for improving the cell protection which comprises the administration of a combination of the extracts of the plants *Vitis vinifera* and *Lycopersicon esculentum* with the vitamins C, E, beta-carotene and optionally selenium. Said combination can be used in the prevention of pathological conditions related in part to an overproduction of free radicals like ageing, arteriosclerosis and cancer.

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METHOD FOR IMPROVING THE CELL PROTECTION

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FIELD OF THE INVENTION

The present invention is related to a novel method of improving the cell protection, which utilises the administration of the combination of the extracts of the plants *Vitis vinifera* and *Lycopersicon esculentum* with the vitamins C, E, beta-carotene and selenium, as well as the use of such combination for treating or preventing pathologies related to an overproduction of free radicals like ageing, arteriosclerosis and cancer.

20

DESCRIPTION OF THE PRIOR ART

Antioxidants act to protect components of the body against free radical damage [Harman D.; Holliday R. and M. Meydany – Editor, "Towards Prolongation of healthy life span", Annals of the New York Academy of Sciences, Vol. 854, 1998, New York; Stählin H.B., "The Impact of Antioxidants on Chronic Disease in Ageing and Old Age", Int. J. Vitamin. Nutr. Res. 69, 146-149, 1999].

Several epidemiological studies support the observation that an increase in antioxidant intake limits the clinical expression of coronary artery diseases and some tumours [Comstock GW; Alberg AJ; Huang HY, Wu K.; Burke AE; Hoffman SC; Norkus EP; Gross M.; Cutler RG; Morris JS; Spate VL; Helzlsouer KJ, "The risk of developing lung cancer associated with antioxidants in the blood: ascorbic acid, carotenoids, alpha-

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tocopherol, selenium, and total peroxy radical absorbing capacity", *Cancer Epidemiol Biomarkers Prev* 6, 907-916, 1997]. Individuals with high dietary intakes of antioxidants may have a greater life expectancy.

5 Individual conditions, environmental stress and oxidative stress induced by environmental and nutritional factors exert its influence on a wide variety of individuals with varying defence capacities.

In general terms, individuals with very well balanced defence mechanisms will remain free of chronic disease, but will eventually succumb to the overall ageing processes.

10 Much more likely, however, is the case where critical systems are insufficiently protected and become injured. The sequence of this process will be clinically diagnosed as chronic disease.

Antioxidants as important elements in the body's defence against oxidative stress thus have a general anti-ageing property as well as a specific disease-protective function.

15 Since the consequences of chronic disease are among the leading causes of disability in old age, primary prevention by adequate antioxidative defence has not only an „anti-ageing“ function, but more importantly, a specific chronic-disease-preventing function. However, it becomes clear that this is a life-long important protective mechanism: primary prevention of age-related chronic diseases has to start early in life and needs to
20 be continued throughout the whole life span.

The relationship between antioxidants and chronic disease is probably best investigated in cardiovascular disease. A strong inverse relationship between plasma antioxidant concentration, morbidity, and mortality from coronary heart disease was demonstrated.

25 The analyses of the data allowed to quantify the contribution of the different antioxidative vitamins. According to these results a sufficient intake of vitamin E is most important, followed by vitamin C and carotene intake.

In the analysis of a population with an overall very good vitamin E status, a strong effect of vitamin C and beta-carotene was observed. It is quite well known that other
30 antioxidants without vitamin functions originating from food, such as flavonoids, polyphenols, or lycopene have very important antioxidative functions [Fuhrman B.; Ben-Yaish L.; Attias J.; Hayek T.; Avaram M., "Tomato lycopene and beta-carotene inhibit low-density lipoprotein oxidation and this effect depends on the lipoprotein

vitamin E content", *Nutr Metab Cardiovasc Dis* 7, 433-443, 1997; Pastori M.; Pfander H.; Boscoboinik D.; Azzi A., "Lycopene in association with alpha-tocopherol inhibits at physiological concentrations proliferation of prostate carcinoma cells", *Biochem Biophys Res Commun* 250 (3), 582-585, 1998; Boehm F.; Edge R.; McGarvey DJ;
5 Truscott TG, "Beta-carotene with vitamins E and C offers synergistic cell protection against Nox", *FEBS Lett* 436, 387-389, 1998; Watkins T.R. – Editor, "Wine, Nutritional and Therapeutic Benefits", American Chemical Society Symposium Series 661, 1997, Washington DC].

10 In late adulthood and early old age, cancer is the leading cause of premature death. There is no question that a large majority of cancers is the result of environmental factors challenging the specific genetic endowment of tissue at high risk for proliferative disorders. Epidemiological evidence clearly demonstrates that a low intake of fruit and vegetables and low plasma concentrations of antioxidative vitamins
15 correlated with a significantly elevated cancer morbidity and mortality. This holds true for gastrointestinal cancer and lung cancer, but also for prostate cancer and for breast cancer.

The neoplastic process is a highly complex sequence of events. Many potentially
20 dangerous mutations occur continuously. Once these genetic changes have escaped the initial defence and repair mechanisms, these altered cells have to be eliminated by other defence mechanisms. One important mechanism is the apoptotic process. It may be that committed cells in the proliferative stage are protected by antioxidants against the body's own immune and apoptotic defence mechanisms. This might explain why
25 antioxidants occur in higher concentration in breast cancer tissue. There is no doubt that a life-long high intake of a diet rich in fruit and vegetables and high in a variety of antioxidants substantially and significantly lowers the risk of malignant disorders in later life.

30 Primary degenerative brain disease and diseases related to cerebral vascular disturbances are the leading cause of disability in old age. Loss of autonomy, dependence and high social costs for individuals and society are the consequences of brain dysfunction. The impressive gain in individual life expectancy is overshadowed

by the loss of mental function in old age. Hence, prevention and treatment of disorders leading to mental impairment have emerged as major challenge of the modern health care system. As in the previous examples, a combination of age-related functional changes superimposed by disorders of circulation or of certain neuronal systems leading to distinct chronic diseases are being observed.

Given the importance of antioxidants in the maintenance of cell integrity and cell function, antioxidants (vitamin E, carotenes, vitamin C) and other micronutrients from the food, e.g. polyphenols, flavonoids, lycopene, may decrease the risk of vascular disease, protect the neurons against oxidative stress and thus maintain neuronal function. Several epidemiological studies in elderly people have revealed a correlation between antioxidants and cognitive performance.

Other epidemiological data have shown that red wine may reduce the mortality rate from heart disease, the so called „French paradox“. An analysis conducted on 34'014 men between 1978 and 1983 in Eastern France has shown that a moderate intake of wine (2-5 glasses of wine) was associated with a 24-31% reduction in all-cause mortality [Renaud S.C. et al., "Alcohol and Mortality in Middle-Aged Men from Eastern France", *Epidemiology* 9, 184-188, 1998].

Although many studies have been conducted with single antioxidant compounds, the pharmacological effects of the administration of the combination of antioxidant substances of natural origin as plant extracts of *Vitis vinifera* (Grape), of *Lycopersicon esculentum* (Tomato) with the vitamins C, E, beta-carotene and selenium have never been studied.

The Israeli Patent Application IL 121112 (1997/06/19) describes a synergistic mixture containing lycopene and vitamin E and its use in the prevention of LDL oxidation. The applicant also published the results in a scientific paper describing that the LDL protection by lycopene exceeded the protection by β -carotene. However, this effect was selective only to LDL's with high vitamin E content and was potentiated when the carotenoids were present in combination with vitamin E [Fuhrman B.; Ben-Yaish L.; Attias J.; Hayek T.; Avaram M., loc. cit.].

The US Patent 5,648,377 (1997/07/15) relates to the combination of lycopene with an extract of *Vitis vinifera* showing that the combination exerts a synergistic antioxidant action.

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SUMMARY OF THE INVENTION

The object of this invention is the surprising discovery that the administration of the combination of the extracts of the plants *Vitis vinifera* and *Lycopersicon esculentum* combined with vitamin C, vitamin E, β -carotene and optionally selenium significantly increases, in a synergistic manner, the cell protection. The results obtained from the described experimental tests show that the improvement of the cell protection with the combination of the mentioned substances are statistically greater than the combination of an extract of *Vitis vinifera* with lycopene (US Patent 5,648,377) or the combination of vitamin E with lycopene (IL Patent application 121112).

The invention therefore relates to a improved method of protecting the cell from a disease caused by an overproduction of free radicals, said method comprises administering a protective or therapeutically effective amount of a composition comprising synergistic amounts of a lipophilic antioxidant and a hydrophilic antioxidant and optionally an acceptable carrier, the improvement wherein is that said lipophilic antioxidant is a mixture consisting essentially of vitamin E, beta carotene and an extract of *Lycopersicum esculentum*, and said hydrophylic antioxidant is a mixture consisting essentially of vitamin C and an extract of *Vitis vinifera*.

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In another aspect, the present invention provides a method of treating a subject suffering from a disease, which is caused by an overproduction of free radicals, comprising the administration of a therapeutically effective amount of a composition according to the present invention.

In a further aspect, the present invention provides a method of treating a subject suffering from an inflammation.

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In another aspect, the method according to the present invention is applicable to the treatment of a subject suffering from atherosclerosis.

In yet another aspect, according to the invention there is provided a method for preventing the mutagenic activity induced by free radicals in a subject.

In still another aspect, the method of the present invention is useful for treating a subject suffering from a tumor.

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DETAILED DESCRIPTION OF THE INVENTION

- 10 In a preferred embodiment the method according to this invention includes the administration of vitamin E, beta carotene, an extract of *Lycopersicum esculentum*, vitamin C, an extract of *Vitis vinifera* and a selenium compound. Preferred selenium compounds are sodium selenite or selenium yeast.
- 15 The term "extract of *Lycopersicum esculentum*" as used hereinabove or hereinbelow includes a dilute composition of lycopene. Said lycopene may be obtained by extraction from plants, algae, fungi or genetically modified organisms or by synthesis. Preferably the extract from dried skin of tomatos is directly utilized.
- The term "extract of *Vitis vinifera* " as used hereinabove or hereinbelow includes a
20 dilute composition procyanolic oligomers. Preferably the extract from the seeds of red vines is directly utilized.
- In another preferred embodiment the method according to this invention includes the administration of
- (a) 5 to 20 parts, preferably 7.5 to 15 parts of vitamin E, in particular vitamin E
25 acetate or vitamin E succinate;
 - (b) 1 to 15 parts, preferably 2 to 10 parts of beta-carotene;
 - (c) 1 to 4 parts, preferably 1.5 to 3 parts of in the form of an extract of
Lycopersicum esculentum obtained from dried skin of tomatoes.
 - (d) 2 to 60 parts, preferably 5 to 50 parts of vitamin C, in particular the sodium salt
30 thereof; and
 - (e) 1 to 30 parts, preferably 1.1 to 25 parts of *Vitis vinifera* extract in particular obtained from the seeds of red vines.

Preferably the lipophilic antioxidant and the hydrophilic antioxidant are administered in the presence of a pharmaceutically acceptable carrier. Suitable excipients are exemplified in: Remington: the science and practice of pharmacy. 19th ed. Easton : Mack Publ., 1995.

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Most preferred carriers are selected from the group consisting of natural plant oils, fully or partially hydrogenated plant oils, in particular soybean oil, fully or partially hydrogenated soybean oil, rape oil, peanut oil, lecithins, in particular soylecithin or egglecithin, plant phosphatides, in particular soyphosphatide and natural waxes, in particular beeswax.

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The compositions may be formulated as solids or solutions. Solid formulations may be for preparation of a solution before injection. Preferably, the pharmaceutical compositions of the invention are administered in the form of soft or hard gelatin capsule, tablet, coated tablet, suppository or in the transdermal form. The dosage will be adjusted according to factors like body weight and health status of the patient, nature of the underlying disease, therapeutic window of the compound to be applied, solubility, and the like. It is within the knowledge of the expert to adjust dosage appropriately.

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Accordingly, a further aspect of the present invention is the use of a composition of the invention in the preparation of a pharmaceutical composition for the treatment of a subject suffering from pathological conditions being caused at least in parts to an overproduction of free radicals like ageing, inflammation, atherosclerosis or cancer. Furthermore, an aspect of the invention is a method of prevention and/or treatment of inflammation, atherosclerosis or cancer, comprising administering an effective amount of a pharmaceutical composition of the invention to a patient.

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The objective of this study was to evaluate first in rat erythrocytes the protective effect of the combination of the extracts of *Vitis vinifera*, *Lycopersicon esculentum*, vitamin C, vitamin E and beta-carotene against the UVB-induced damage and the existence of a cooperative interaction between its components, and then in keratinocytes cell lines the ability of selenium to increase the endogenous enzymatic antioxidant defences.

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In detail the following investigations have been carried out:

- a) the dose-dependent ability of the combination of the extract of *Vitis vinifera*, the extract of *Lycopersicon esculentum*, vitamin C, vitamin E and beta-carotene, thereafter denominated in this text as "combination of the active ingredients", to prevent hemolysis induced by large UVB doses (1.5 – 7.0 J/cm²);
- b) the dose-dependent ability of a mixture of the lipophilic antioxidants (*Lycopersicon esculentum* extract, beta-carotene + vitamin E acetate) to inhibit hemolysis and lipid peroxidation in red cell membranes;
- c) the dose-dependent ability of a mixture of the hydrophilic antioxidants (*Vitis vinifera* extract and vitamin C) to inhibit hemolysis and lipid peroxidation in red cell membranes;
- d) the protective effect on hemolysis and lipid peroxidation in red cell membranes by the association of the mentioned lipophilic and hydrophilic antioxidants;
- e) the ability of selenium yeast to release selenium in a cellular system (keratinocytes): induction of glutathione peroxidase (GSH-PX) activity and cellular resistance to oxidative stress induced by physical (UVB) or chemical (cumene hydroperoxide) cytotoxic agents.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: shows the dose-dependent protective effect of the combination of the active ingredients on UVB-induced hemolysis (Example 1)

Figure 2: shows the dose-dependent protective effect of lipophilic components of the combination of the active ingredients on UVB-induced hemolysis (Example 2)

Figure 3: shows the dose-dependent protective effect of hydrophilic components of the combination of the active ingredients on UVB-induced hemolysis (Example 2)

Figure 4: shows the UVB-induced hemolysis: cooperative antioxidant interaction between lipophilic and hydrophilic components of the combination of the active ingredients (Example 3)

5 Figure 5: shows the protective effect of the combination of the active ingredients on RBC hemolysis induced by 120 min irradiation (Example 3)

Figure 6: shows the effect of selenium yeast and sodium selenite supplementation on keratinocytes viability (Example 4)

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Figure 7: shows the effect of selenium yeast and sodium selenite supplementation on keratinocyte glutathione peroxidase activity (Example 5)

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Figure 8: shows the effect of selenium yeast and sodium selenite supplementation on glutathione peroxidase activity in keratinocytes exposed to UVB radiation (50 mJ/cm²) (Example 5)

Figure 9: shows the effect of selenium yeast and sodium selenite supplementation on cumene hydroperoxide (CuOOH)-induced cell death (Example 6)

20

Figure 10: shows the effect of selenium yeast and sodium selenite supplementation on CuOOH-induced intracellular oxidative stress (DCF formation) (Example 7)

The following abbreviations have been used in Figures 7 to 10:

Yeast (L)	Selenium yeast (3.95 µg/ml): 50 nM Se
Yeast (H)	Selenium yeast (39.5 µg/ml): 500 nM Se
Selenite (L)	Na ₂ SeO ₃ 50 nM
Selenite (H)	Na ₂ SeO ₃ 500 nM

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One skilled in the art will appreciate that although specific reagents and reaction conditions are outlined in the following examples, modifications can be made which are meant to be encompassed by the scope of the invention. The following examples, therefore, are intended to further illustrate the invention and are not limiting.

EXAMPLES

5 MATERIALS AND METHODS

Chemicals

The used organic solvents were of analytical grade (Carlo Erba, Milan, Italy). The active ingredients (*Vitis vinifera* extract, *Lycopersicon esculentum* extract, beta-carotene, lycopene, d,l- α tocopherol acetate, ascorbic acid, selenium yeast) were
10 supplied by Pharmaton SA, CH 6934 Bioggio, Switzerland. Cumene hydroperoxide (CuOOH), thiobarbituric acid (TBA), EDTA, sodium selenite (Na₂SeO₃), sodium azide (NaN₃), sodium pyruvate, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), DMEM (Dulbecco's modified Eagle's medium), phenol red free DMEM, L-glutamine,
15 penicillinstreptomycin solution were purchased from Sigma (Trinital, Milan, Italy); fetal calf serum and phosphate buffer solutions (PBS) from Hyclone (Belbio, Milan, Italy); H₂O₂ (30% v/v) from Fluka (Trinital, Milan, Italy); NADH, NADPH, GSH and GSH-reductase from Boehringer Mannheim Italy (Milan, Italy).

Apparatus

20 Spectrophotometric and fluorimetric studies were carried out in a computer-aided Perkin Elmer Lambda 16 spectrophotometer and a computer aided Perkin Elmer LS50B luminescence spectrometer (Perkin Elmer, Monza, Italy).

UVB irradiation of erythrocytes (RBC)

25 Erythrocytes from male Wistar rats (Charles River, Calco, Italy), isolated and washed from white blood cells and platelets were suspended in phosphate buffered saline, pH 7.4 [125 mM NaCl and 10 mM sodium phosphate buffer (PBS)], stored at 4°C and used until 4 days old. For experiments, an aliquot of the erythrocyte suspension was drawn
30 from the stock, centrifuged at 1000 x g for 5 min and 0.1 ml of the pellet diluted to 50 ml with PBS obtain a 1% erythrocyte suspension (approximately 15 x 10⁷ cells/ml). 50 ml aliquots of the RBC suspension were placed in Pyrex glass dishes (14 cm diameter) transparent to exciting-light wavelengths > 290 nm and exposed to UVB

radiation for different time intervals. When the inhibitors were added to the RBC suspension, a pre-incubation period of 30 min at 37°C was applied. UVB radiation was carried out with a parallel bank of two Philips TL20W/12 fluorescence tubes (Sara s.r.l., Castellanza, Italy) emitting a continuous spectrum between 280 and 320 nm with a peak emission at 312 nm. Fluence rate at the site of cell irradiation (25 cm from UVB source) was of 0.85 mW/cm² and the UVB doses employed ranged from 1.5 to 7.0 J/cm² (exposure time 30-150 min), as measured with a Vilber Lourmat VLX-3W radiometer (UVB probe, 312 nm).

10 UVB-induced hemolysis

After irradiation, 2 ml aliquots of the RBC suspension were diluted 1:5 with buffered saline and hemolysis determined turbidimetrically at 710 nm at 30 min intervals for 180 min. Percentages hemolysis were determined setting as a 100% hemolysis the absorbance value determined in RBC suspensions sonicated for 5 sec at 50% power. Mean values of 5 determinations were used for calculation.

Keratinocytes

The NCTC 2544 human keratinocyte celline (Flow Laboratories, Irvine, UK) was cultured at 37°C in DMEM supplemented with 10% fetal calf serum 2 mM L-glutamine and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin). Cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. For experiments, cells were seeded at a density of 5 x 10⁴/cm² and cultured in 2 cm² cell plastic culture dishes until 80-90% confluence. Cell viability was determined by the trypan blue exclusion assay and by measuring the LDH leakage. Protein determinations were performed by a modified Lowry method, using bovine serum albumin as a standard.

Glutathione peroxidase (GSH-PX) activity

Cells grown were treated with sodium selenite (50, 500 nM) or selenium yeast (3.95 or 39.5 µg/ml corresponding to 50 and 500 nM Se) and incubated to 90% confluence (48 h). Before irradiation cell monolayers were washed three times with prewarmed PBS and exposed to UVB (50 mJ/cm²), in the presence of PBS. After irradiation, PBS was replaced by serum-phenol red free DMEM (K-DMEM) and incubated for 24 h (sham-control cells were subjected to the same procedure without UV exposure). The media

were discarded and the cells removed by gently scraping into 1 ml of 50 mM Tris-HCl buffer and lysed by sonication. The cellular suspensions (0.1 ml aliquots) were analysed for GPX activity by monitoring the decrease of absorbance at 340 nm for the rate of disappearance of NADPH in a thermostated (37°C) spectrophotometer. GPX activity was calculated using a molar extinction coefficient of NADPH of $6.22 \times 10^3 \text{ cm}^{-1} \text{ m}^{-1}$ and expressed as mU/mg protein, where 1 U of enzyme activity is defined as μmoles substrate consumed or product produced/min at 37°C.

CuOOH-induced oxidative stress

The intracellular oxidative stress induced by CuOOH and the protective effect by selenium yeast was determined by measuring cell viability and peroxide content by using the oxidation-sensitive fluorescent probe DCF. Cells grown were treated with sodium selenite (50, 500 nM) or selenium yeast (3.95 or 39.5 $\mu\text{g/ml}$ corresponding to 50 and 500 nM Se) and incubated to 90% confluence (48 h). Stock solution of DCFH-DA (3.34 mM) were prepared in ethanol, purged with nitrogen and stored at -20°C in 500 μl aliquots. Cell monolayers were washed with prewarmed PBS and incubated with 10 μM DCFH-DA in PBS for 30 min at 37°C. Thereafter the medium was discarded and the cell monolayer washed three times with prewarmed PBS to remove not-incorporated DCFH-DA and then incubated with K-DMEM containing 0.5 mM CuOOH. Sham-control cells were subjected to the same procedure without CuOOH exposure. After 24 h incubation, the media were discarded and the cells removed by gently scraping into 3 ml of PBS. The cellular suspension was then transferred into 1 ml thermostated fluorescence cuvettes (25°C) equipped with a magnetic stirrer and the increase of fluorescence intensity of the probe (λ_{exo} 502 nm; λ_{em} 520 nm; band widths 5 nm) recorded. The fluorescence intensity values at all the observation times were correlated to protein content (means \pm S.D. of 6 determinations) and expressed as % increase of fluorescence in respect to the controls (sham-control cells).

Statistical analysis

Results are expressed as mean \pm S.D. of 5 independent determinations. Data were analysed by one-way analysis of variance (ANOVA), followed by the Tukey's test for statistical significance among groups and by Student's *t*-test (two tailed) for unpaired

samples. Differences were considered significant when $p < 0.05$. Statistical analysis was performed by using the Prism software package (GraphPad Software Inc., San Diego CA, USA).

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Example 1: ERYTHROCYTES AS CELL MODEL

Protective effect of the combination of the active ingredients

Exposure of rat RBC to increasing UVB doses gives the typical hemolysis curve reported in Figure 1: the hemolytic process begins from 60 min irradiation, corresponding to an UVB dose of approximately 3 J/cm² ($8.5 \pm 3.2\%$) and reached the plateau between 90 ($87.2 \pm 6.3\%$) and 120 minutes ($98.7 \pm 4.4\%$). The samples of the combination of active ingredients were prepared by mixing with PBS and vortexed; the homogenous suspension obtained was further diluted in PBS to final concentrations ranging from 0.1-10 µg/ml. When unirradiated RBC were incubated under aerobic conditions in the presence of the combination of active ingredients no significant hemolysis was observed within 3 h. The combination of active ingredients added to RBC suspension before irradiation, markedly and dose-dependently protected RBC from UVB-induced hemolysis (Figure 1), with a minimal effective concentration of 0.1 µg/ml. At 5 µg/ml the hemolytic process was delayed by 30 min, and after 120 min irradiation the percentage hemolysis was only $34.5 \pm 5.5\%$ and at the highest concentration (10 µg/ml) RBC integrity was maintained until 150 min UVB irradiation (7 J/cm²). The IC₅₀ value, calculated at 120 min, was 4 µg/ml.

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Example 2: *Protective effect of the combination of lipophilic and hydrophilic antioxidants of the combination*

For a better understanding of the protective effect of the combination of the active ingredients on UVB-induced hemolysis, we have investigated the radical scavenging ability of a mixture of lipophilic (vitamin E acetate + beta-carotene + extract of *Lycopersicon esculentum*) and hydrophilic antioxidants (vitamin C + extract of *Vitis vinifera*).

30

Stock solutions of vitamin E acetate, beta-carotene and extract of *Lycopersicon*
esculentum were prepared in THF under N₂ atmosphere and red light to prevent
autooxidation and stored at -20°C up to 1 week. The stability of each antioxidant was
daily checked by UV spectroscopy. In order to maintain the same ratios between the
antioxidants present in the combination of active ingredients, a mixed stock solution of
5 7.04 mg/ml for vitamin E acetate, 1.64 mg/ml for beta-carotene and of extract of
Lycopersicon esculentum corresponding to 0.88 mg/ml lycopene was prepared by
dilution with THF. A serial dilution was made to give the mixed lipophilic antioxidant
working solutions at final concentrations of 1.2, 2.4, 4.8, 9.6 µg/ml of RBC suspension
10 (total lipophilic antioxidants). In all the experiments THF concentration was always
0.1% (v/v).

The same approach was used for hydrophilic antioxidants: a mixed stock solution of
vitamin C (6.72 mg/ml) and extract of *Vitis vinifera* (2.8 mg/ml) was prepared in PBS
15 and a serial dilution was made to give the mixed hydrophilic antioxidant working
solutions at 0.58, 1.16, 2.32, 4.64, 9.28 µg/ml RBC suspension (total hydrophilic
antioxidants).

As shown in Figures 2 and 3, both the lipophilic and the hydrophilic fractions of the
combination of active ingredients are able to prevent UVB-induced hemolysis, in a
20 dose-dependent manner, although with different potency. The greater activity of the
hydrophilic mixture is mainly due to the presence of the *Vitis vinifera* extract, which
has been shown in different experimental models in vitro to be a potent chain-breaking
antioxidant (Maffei Facino R.; Carini M.; Aldini G.; Bombardelli E.; Morazzoni P.;
Morelli R.; Free radicals scavenging action and anti-enzyme activities of Procyanidines
25 from *Vitis vinifera* – A mechanism for their capillary protective action; Arzneim.
Forsch./ Drug Res., 44, 592-601, 1994) and to save endogenous vitamin E from UVB-
induced consumption, through recycling mechanism (hydrogen transferring reaction).

Examlpe 3: *Cooperative antioxidant interaction between lipophilic and*
30 *hydrophilic antioxidants of the combination*

The cooperative antioxidant interaction between lipophilic and hydrophilic antioxidants
of the combination was studied by using concentrations of each mixed working solution
that give approximately 20-30% inhibition at 120 min irradiation: 2.4 µg/ml for

lipophilic (Vitamin E acetate 1.76 µg/ml, β-Carotene 0.41 µg/ml, lycopene 0.22 µg/ml) and 0.58 µg/ml for hydrophilic (Vitamin C 0.4 µg/ml, extract of *vitis vinifera* 0.18 µg/ml). The results are shown in Figures 4 and 5. Under these conditions, the rate of hemolysis was similar for both the mixtures, the hydrophilic being slightly more active than the lipophilic one (20% vs 14% inhibition at 120 min). When the antioxidants were combined, the rate of hemolysis was markedly slowed and the integrity of RBC was maintained up to 90 min. At 120 min the percentage inhibition ($55 \pm 2.5\%$) was far beyond the sum of the % inhibitions (calculated: 33.8%) observed with the individual antioxidant mixtures, to indicate a synergistic interaction. The protective effect elicited by a typical combination of hydrophilic/lipophilic antioxidants (*Vitis vinifera* extract + lycopene) did not differ significantly from that observed with the combination of the hydrophilic ingredients (Figure 5); by contrast, the association between vitamin E (1.76 µg/ml) and lycopene (0.22 µg/ml) gives a percentage inhibition of hemolysis significantly lower than that obtained with the combination of the lipophilic ingredients (7.1% vs 14%) (Figure 5).

Example 4: *Effect of selenium yeast supplementation on cell growth and cell viability*

In cultured keratinocytes, sodium selenite or selenium yeast had no apparent growth-stimulating activity. Cell viability, determined LDH leakage (Figure 6), was always over 95% and not significantly different in respect to the sham-control cells at 50 and 500 nM Se doses.

Example 5 *Effect of selenium yeast supplementation on GSH-PX activity*

Figure 7 shows the effects of selenium yeast or sodium selenite supplementation (equimolar Se doses) in control cells: with sodium selenite maximal induction of GSH-PX activity was found at 50 nM (3.365 fold increase vs controls; 91.44 ± 10.37 vs 28.31 ± 5.87 mU/mg protein), and no further increase was observed at 500 nM concentration. Selenium yeast was less potent as GSH-PX inducer at the lower concentration (2.44-fold increase; 63.05 ± 5.51 mU/mg protein), but at the concentration 39.5 µg/ml (corresponding to 500 nM Se) the increase in the enzymatic activity was comparable to that obtained with the equimolar dose of sodium selenite (90.11 ± 10.35 mU/mg

protein). These results clearly demonstrate that selenium yeast dose-dependently releases selenium for enzyme activation.

GSH-PX activity was also determined in keratinocytes exposed to UVB irradiation (50 mJ/cm²), and the extent of enzyme induction by selenium yeast or sodium selenite was significantly enhanced in comparison to the control cells (Figure 8), thus to indicate that also under oxidant imbalance selenium is still active.

Example 6: *Effect of selenium yeast supplementation on CuOOH-induced cell death*

Cumene hydroperoxide is a strong promoter of free radical reactions and incubation of keratinocytes with 500 µM CuOOH produced more than 80% cell death 24 h after exposure (Figure 9). In selenium yeast-supplemented cells, cell death was markedly and dose-dependently restrained, cell viability being 64 ± 1.3% at the lowest dose (3.95 µg/ml) and not significantly different from controls cells (90 ± 1.2%) at the highest Se dose (39.5 µg/ml ≈ 500 nM Se). The protection afforded by 50 nM sodium selenite was slightly greater, since cell viability was almost completely recovered.

Example 7: *Effect of selenium yeast supplementation on intracellular CuOOH-induced oxidative stress*

The intracellular oxidative status was determined by the sensitive fluorimetric DCF assay. The assay is based on the ability of the non-polar, non-fluorescent DCFH-DA to diffuse through the cell membrane and to be deacetylated by cytosolic esterases to form the polar, non-fluorescent dichlorodihydrofluorescein (DCFH). This last is trapped within the cytosol where, by reacting with reactive oxygen species generated by CuOOH, gives rise to the formation of the fluorescent derivative dichlorofluorescein (DCF).

Incubation of keratinocytes with 500 µM CuOOH results in a significant accumulation of DCF (maximal after 24 h; more than 7-fold increase), to confirm the spreading of an oxidative burst in the cytosol compartment (Figure 10). Pretreatment of the cells with selenium yeast counteracts the oxidative insult, since a 60% reduction of DCF formation was observed at the lower dose (3.95 µg/ml) and an almost complete

protection at 39.5 µg/ml (550 nM Se). Sodium selenite totally prevents DCF formation already at the lowest dose (50 nM).

5 These results of these examples indicate that it is the association between lipophilic and hydrophilic antioxidants, which is the object of the present invention that provides optimal cell protection against the oxidative challenge of UVB. In other words, there exists a synergistic interaction among antioxidants which explains the high degree of protection obtained with the combination of the active ingredients.

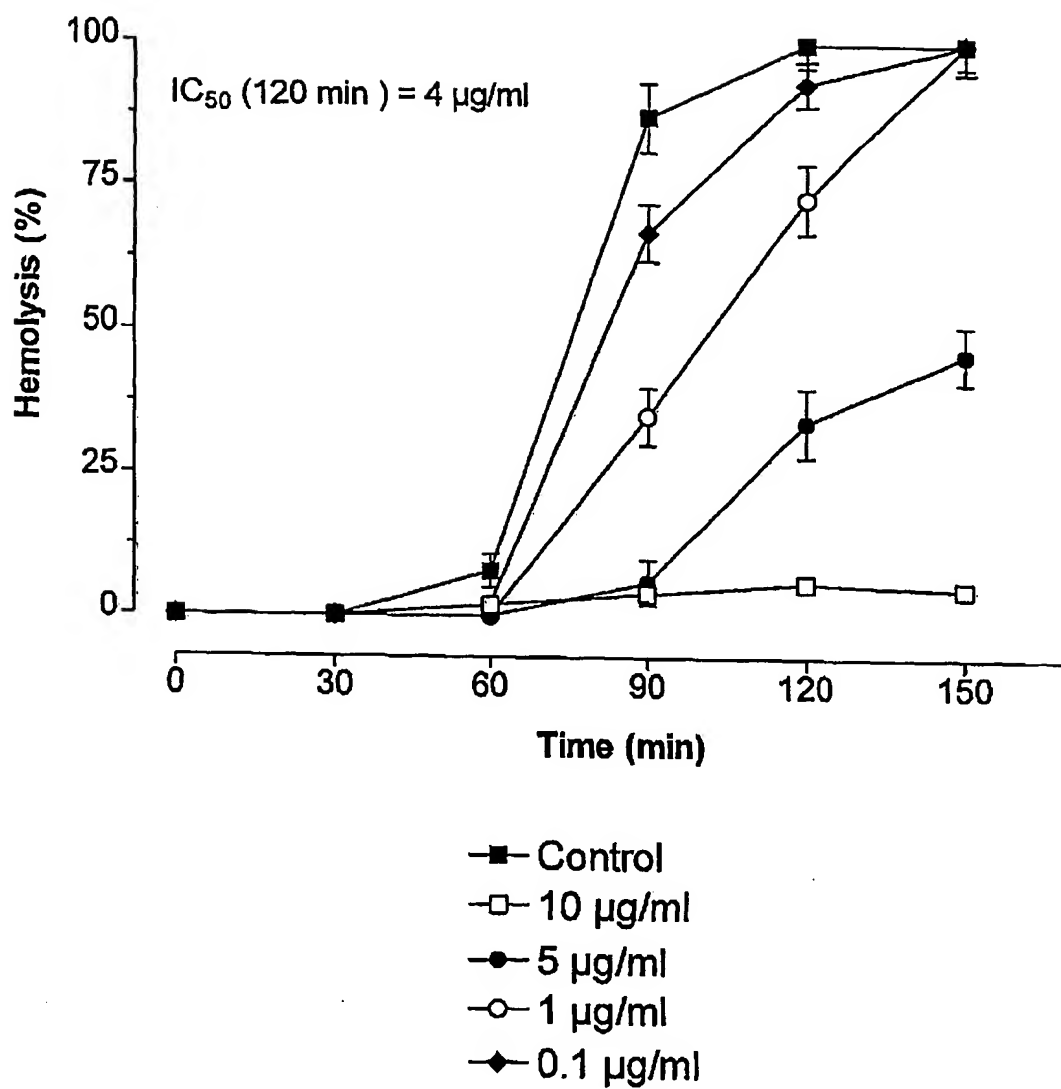
10 Since free radicals and reactive oxygen species (ROS) are generated in both the lipid and aqueous cell compartments, an antioxidant defence system as in the combination of the active ingredients, the object of the present invention, which can partition in both lipid and aqueous environments, is highly suited for cell protection.

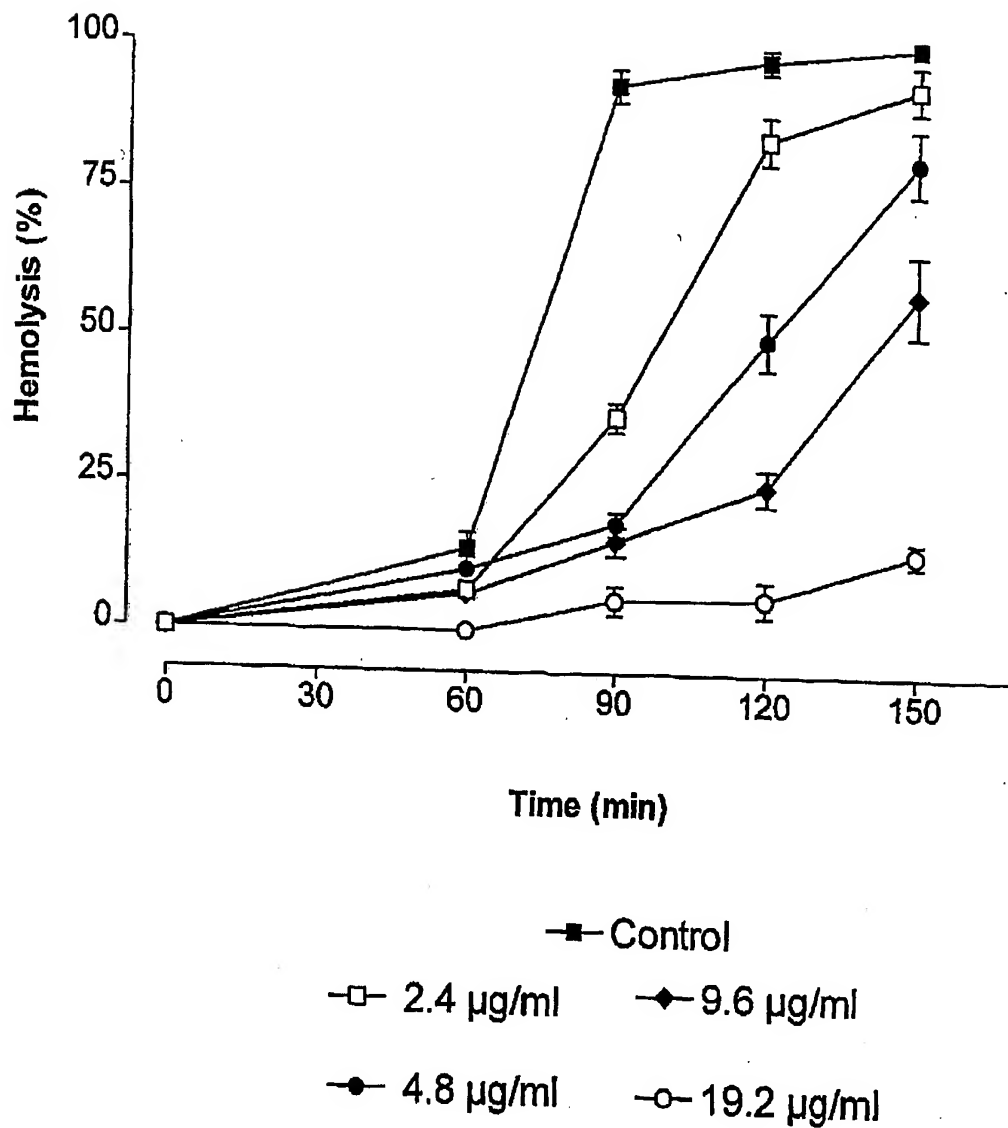
What we claim is:

1. An improved method of protecting the cell from a disease caused by an
5 overproduction of free radicals, said method comprises administering a protective or
therapeutically effective amount of a composition comprising synergistic amounts of a
lipophilic antioxidant and a hydrophilic antioxydant and optionally an acceptable
carrier, the improvement wherein is that said lipophilic antioxydant is a mixture
consisting essentially of vitamin E, beta carotene and an extract of *Lycopersicum*
10 *esculentum* , and said hydrophylic antioxydant is a mixture consisting essentially of
vitamin C and an extract of *Vitis vinifera*.
2. The method according to claim 1 wherein the composition comprises additionally
selenium.
- 15 3. The method according to claim 1 wherein the lipophilic antioxidant comprises
 - (f) 5 to 20 parts of vitamin E;
 - (g) 1 to 15 parts of beta-carotene; and
 - (h) 1 to 4 parts of lycopene in the form of an extract of *Lycopersicum esculentum*.
- 20 4. The method according to claim 1 wherein the hydrophilic antioxidant comprises
 - (i) 2 to 60 parts of vitamin C; and
 - (j) 1 to 30 parts of *Vitis vinifera* extract.
- 25 5. The method according to claim 1 wherein the lipophilic antioxidant comprises vitamin
E acetate or vitamin E succinate.
6. The method according to claim 1 wherein the hydrophilic antioxidant comprises the
sodium salt of vitamin C.
- 30 7. The method composition according to claim 2 wherein the composition comprises
sodium selenite or selenium yeast.

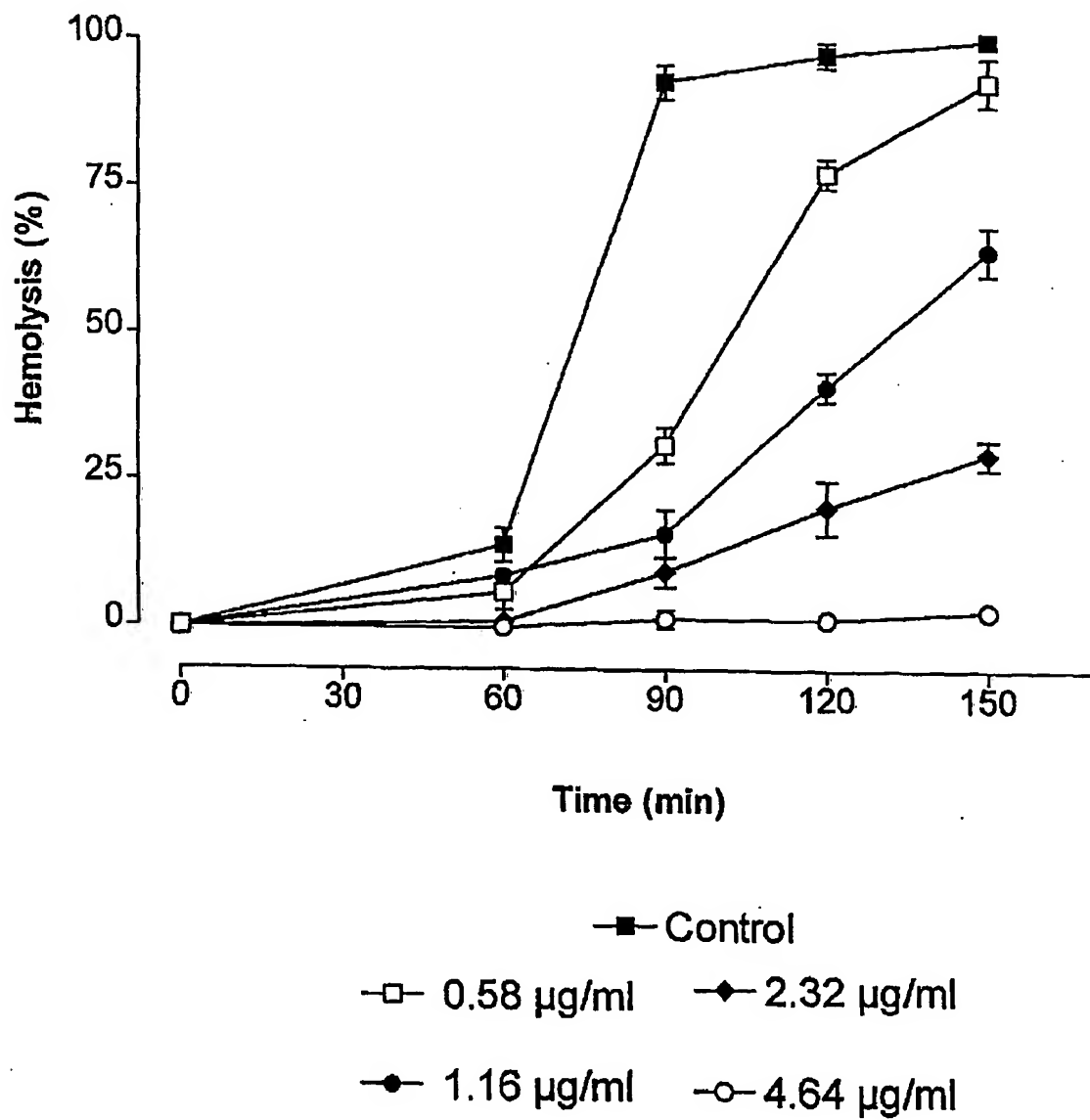
8. The method according to claim 1 wherein said carrier is selected from the group consisting of natural plant oils, fully or partially hydrogenated plant oils, lecithins, plant phosphatides and natural waxes.
- 5 9. The method according to claim 8 wherein said carrier is selected from the group consisting of soybean oil, fully or partially hydrogenated soybean oil, rape oil, peanut oil, soylecithin, soyphosphatide, egglecithin and beeswax .
- 10 10. The method according to claim 1 wherein the composition is administered in the form of soft or hard gelatin capsule, tablet, coated tablet, suppository or in the transdermal form.
- 15 11. An improved method of protecting a subject from suffering from a disease or treating a subject suffering from a disease, said disease being caused by an overproduction of free radicals, said method comprises administering a protective or therapeutically effective amount of a composition comprising synergistic amounts of a lipophilic antioxidant and a hydrophilic antioxydant and optionally an acceptable carrier, the improvement wherein is that said lipophilic antioxydant is a mixture consisting essentially of vitamin E, beta carotene and an extract of *Lycopersicum esculentum* ,
20 and said hydrophylic antioxydant is a mixture consisting essentially of vitamin C and an extract of *Vitis vinifera*.
- 25 12. The method according to claim 11 wherein said subject suffering from pathological conditions being caused at least in parts to an overproduction of free radicals like ageing, inflammation , atherosclerosis or cancer.

1 / 10

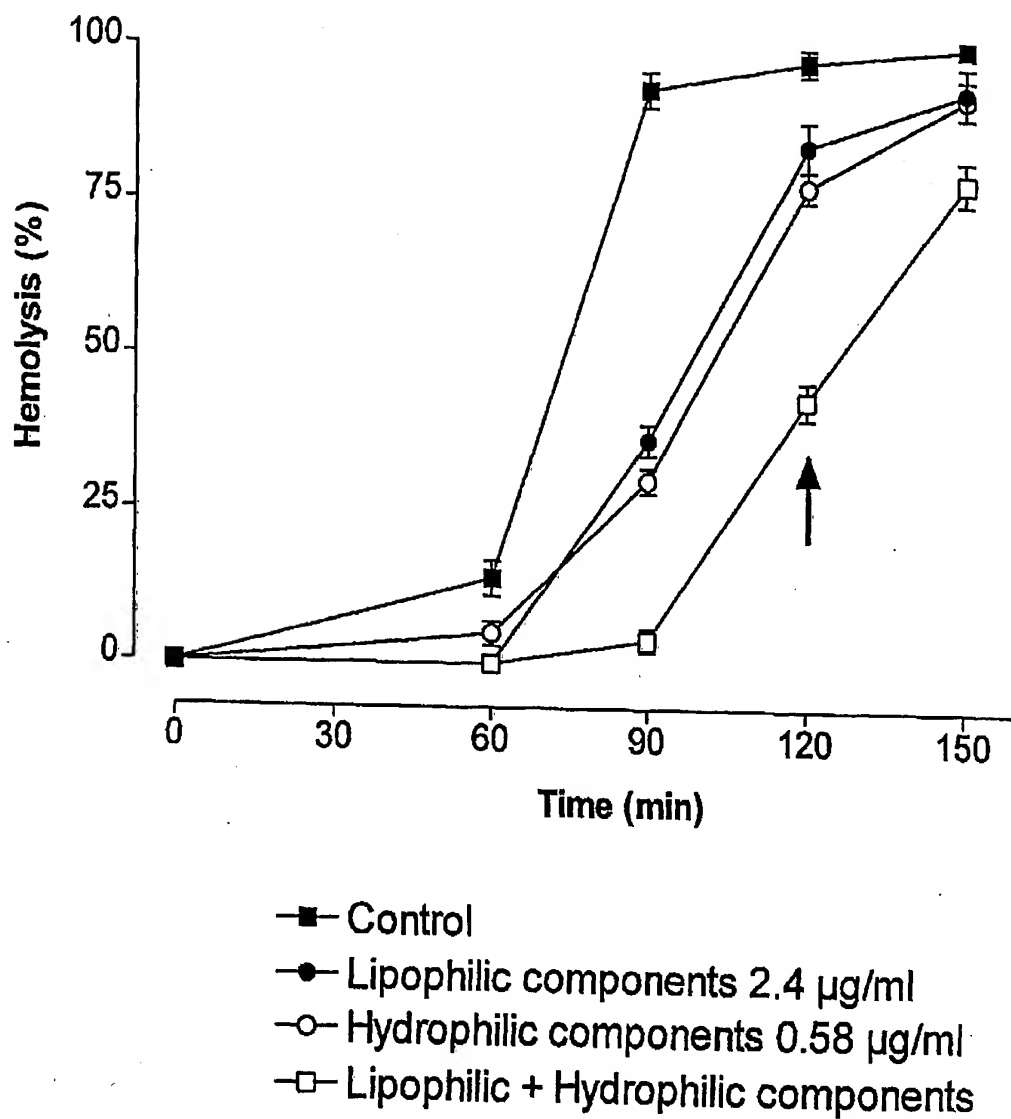
Figure 1

Figure 2

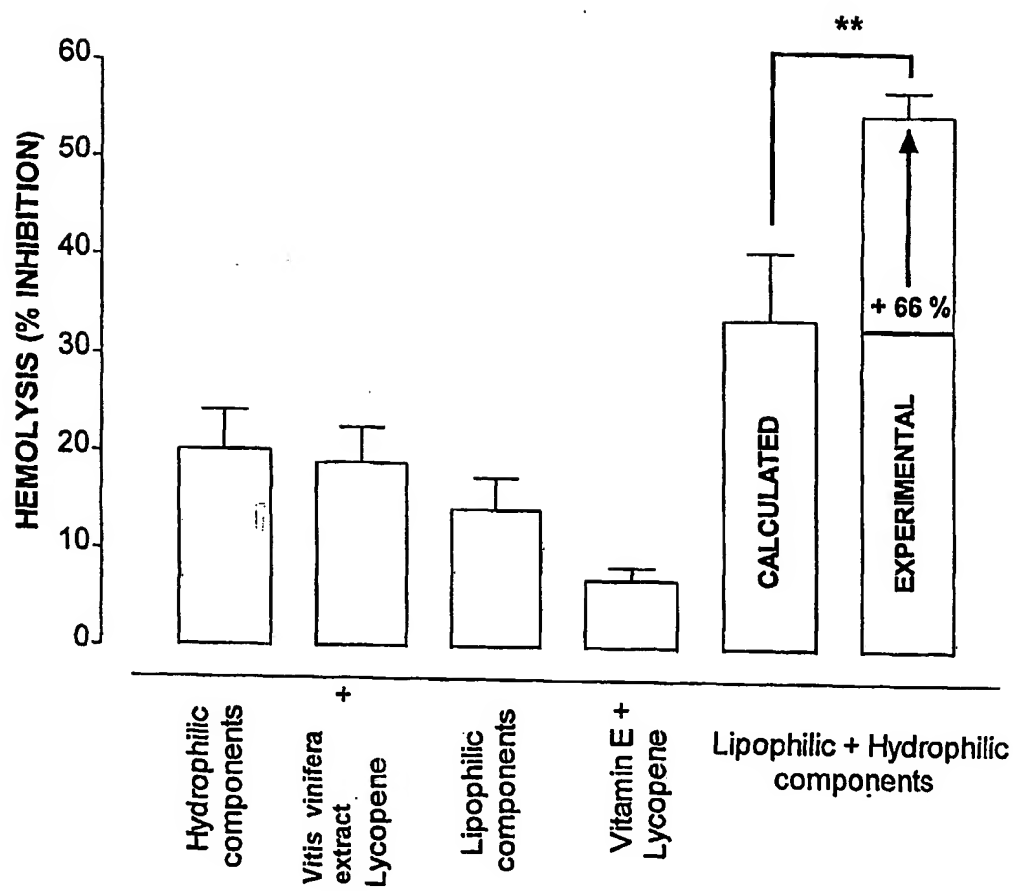
3 / 10

Figure 3

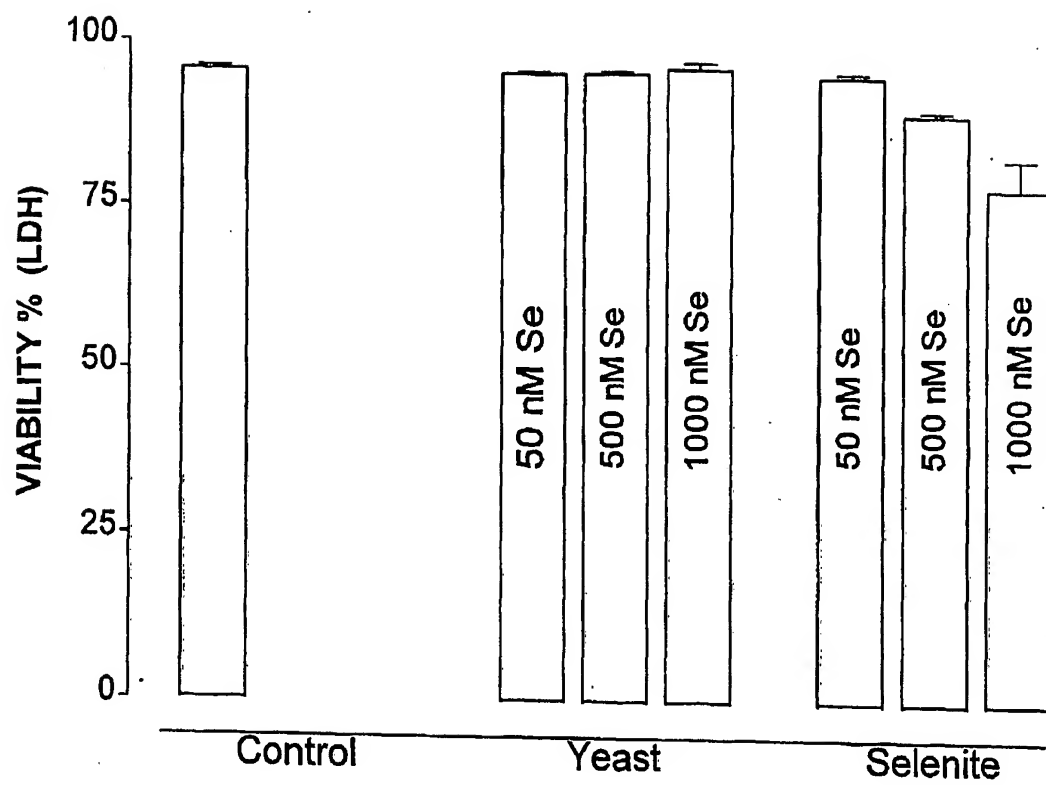
4 / 10

Figure 4

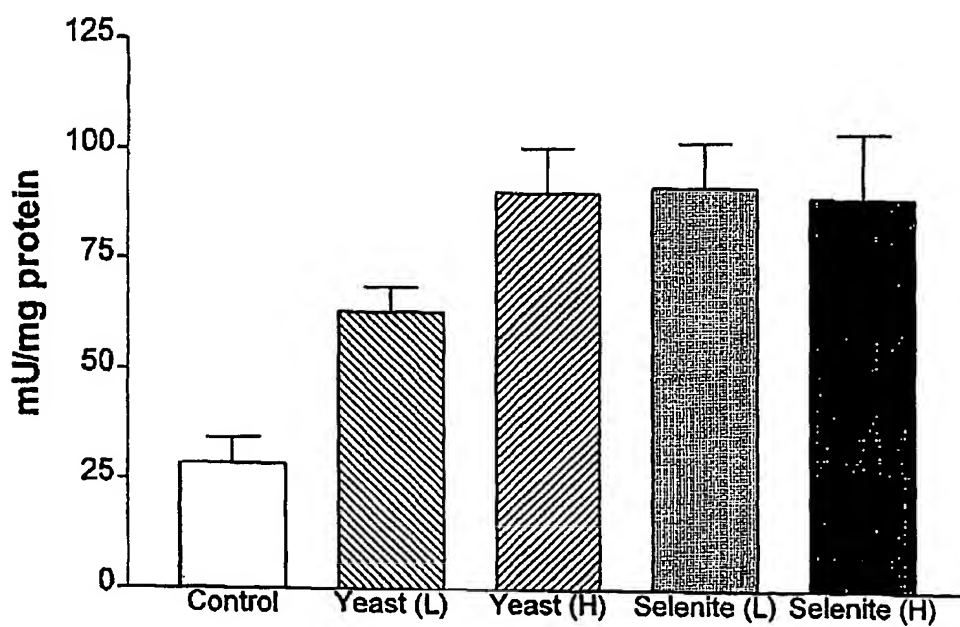
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Figure 5

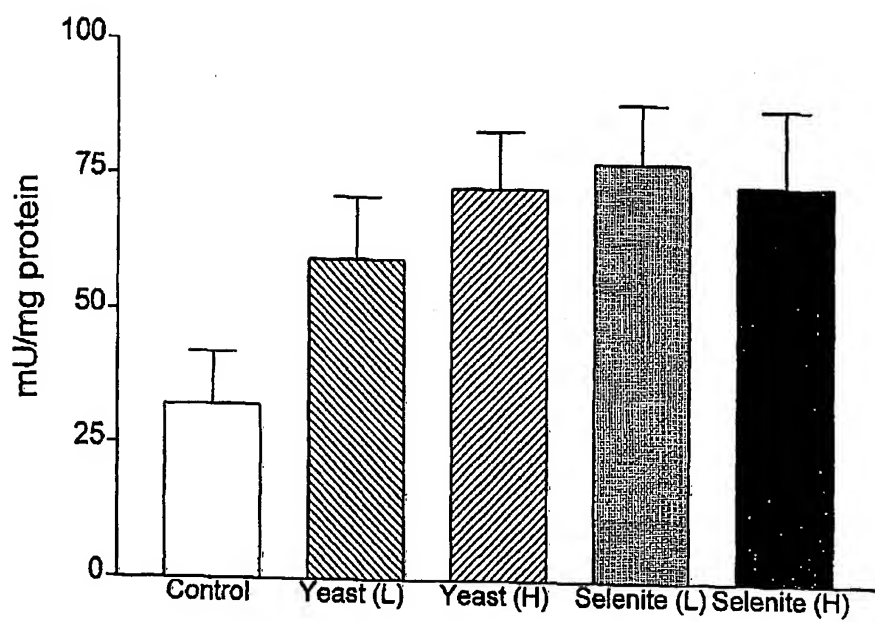
6 / 10

Figure 6

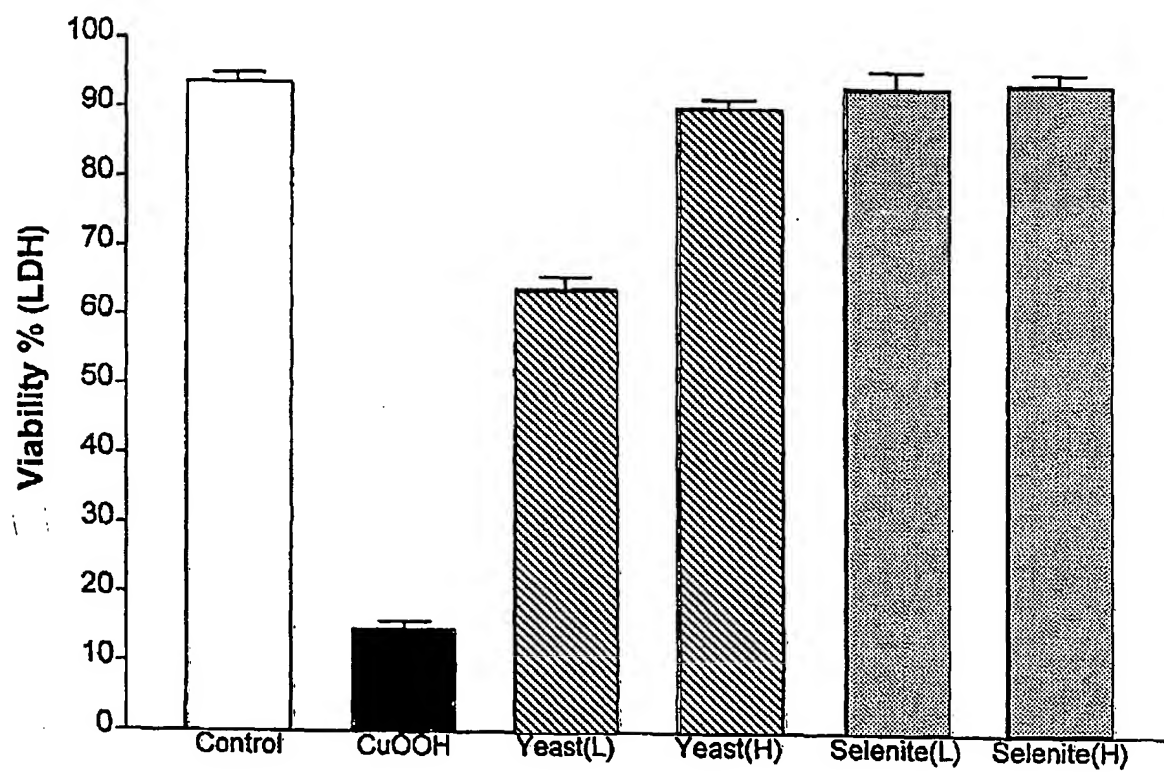
7 / 10

Figure 7

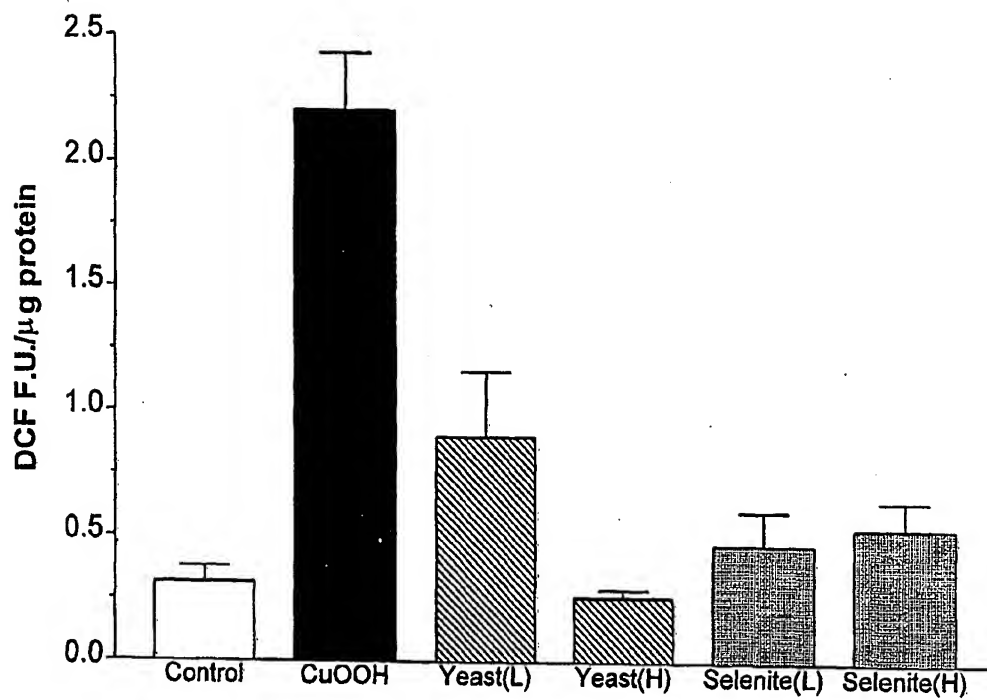
8 / 10

Figure 8

9 / 10

Figure 9

10 / 10

Figure 10

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
29 November 2001 (29.11.2001)

PCT

(10) International Publication Number
WO 01/089542 A3

- (51) International Patent Classification⁷: **A61K 35/78**,
A61P 35/00, 29/00, 9/00, A61K 31/375, 31/355, 33/04 //
(A61K 35/78, 35:74, 33:04, 31:375, 31:355, 31:015)
- (74) Agent: **LAUDIEN, Dieter**; Boehringer Ingelheim GmbH,
55216 Ingelheim am Rhein (DE).
- (21) International Application Number: PCT/EP01/05841
- (81) Designated States (*national*): CA, CN, ID, JP, MX, US.
- (22) International Filing Date: 22 May 2001 (22.05.2001)
- (84) Designated States (*regional*): European patent (AT, BE,
CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC,
NL, PT, SE, TR).
- (25) Filing Language: English
- Published:
— with international search report
- (26) Publication Language: English
- (30) Priority Data: 60/206,945 25 May 2000 (25.05.2000) US
- (88) Date of publication of the international search report:
28 November 2002
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- For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.*
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- (75) Inventor/Applicant (*for US only*): **SOLDATI, Fabio**
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WO 01/089542 A3

(54) Title: COMPOSITION FOR IMPROVING THE CELL PROTECTION COMPRISING A LIPOPHILIC ANTIOXIDANT AND A HYDROPHILIC ANTIOXIDANT

(57) Abstract: The invention relates to a method for improving the cell protection which comprises the administration of a combination of the extracts of the plants *Vitis vinifera* and *Lycopersicon esculentum* with the vitamins C, E, beta-carotene and optionally selenium. Said combination can be used in the prevention of pathological conditions related in part to an overproduction of free radicals like ageing, arteriosclerosis and cancer.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 01/05841

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K35/78 A61P35/00 A61P29/00 A61P9/00 A61K31/375
 A61K31/355 A61K33/04 //(A61K35/78, 35:74, 33:04, 31:375, 31:355,
 31:015)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 48386 A (STUECKLER FRANZ) 30 September 1999 (1999-09-30) abstract; claims 5-7,9,10; examples 1,2,4 page 3, line 26-33 ---	1,3-5, 8-11
X	EP 0 712 630 A (JCB COSMETIQUES) 22 May 1996 (1996-05-22) claims 1,2,7-9,11; examples 1-3 ---	1,2,5, 8-11
P,X	FR 2 792 831 A (BIONATEC SARL) 3 November 2000 (2000-11-03) abstract page 6, line 1,2; claims 7,9,11 ---	1-12
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☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the International filing date but later than the priority date claimed

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X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the International search

8 August 2002

Date of mailing of the International search report

16/08/2002

Name and address of the ISA

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Authorized officer

Gonzalez Ramon, N

INTERNATIONAL SEARCH REPORT

Int # Application No
PCT/EP 01/05841

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	FR 2 799 345 A (GERVAIS DANONE SA) 13 April 2001 (2001-04-13) abstract; claims 3,11,12 page 15, line 5-15 page 10, line 15-20 ----	1-12
Y	US 5 648 377 A (MORAZZONI PAOLO ET AL) 15 July 1997 (1997-07-15) cited in the application abstract; claims 1,9; figure 1 ----	1-12
Y	FUHRMAN B. ET AL: "Lycopene synergistically inhibits LDL oxidation in combination with vitamin E, glabridin, rosmarinic acid, carnosinic acid, or garlic" ANTIOXIDANTS & REDOX SIGNALING, vol. 2, no. 3, 2000, pages 491-506, XP001077095 abstract page 503, column 2 -page 504, column 1 ----	1-12
P,Y	US 6 090 414 A (PASSWATER RICHARD A ET AL) 18 July 2000 (2000-07-18) abstract; claims 1,26,27,61,62 -----	1-12

INTERNATIONAL SEARCH REPORT

tional application No.
PCT/EP 01/05841

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 1-12 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☒ Claims Nos.: 3,4,8
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 3,4,8

Present claim 8 relate to an extremely large number of possible compounds as "carriers". Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible.

Present claims 3,4 relate to a composition defined by reference to the following parameter:
"parts of".

The use of this parameter in the present context is considered to lead to a lack of clarity within the meaning of Article 6 PCT. It is impossible to compare the parameters the applicant has chosen to employ with what is set out in the prior art. The lack of clarity is such as to render a meaningful complete search impossible.

Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the compounds recited in claim 1,5-7,9 and the compositions disclosed in the examples.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 01/05841

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9948386	A	30-09-1999	AT 407821 B AT 52598 A WO 9948386 A1 AU 2911399 A CA 2325437 A1 EP 1065946 A1	25-06-2001 15-11-2000 30-09-1999 18-10-1999 30-09-1999 10-01-2001
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